GenoSensor Corporation

EduPrimer™ VNTR DNA Profiling Kit

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User Manual

EduPrimer™ VNTR DNA Profiling Kit Manual
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Literature Citation

When describing a procedure for publication using these products, we would appreciate that you refer to them as the EduPrimer™ VNTR DNA Profiling Kit.

Trademarks
EduPrimer™ is a trademark of GenoSensor.

Notes for Instructors

Kit Components and Storage Conditions:

Component	Storage
Solution A	Room temp.
Solution B	Room temp.
Cotton Swabs	Room temp.
2X PCR Master Mix	-20ºC
Positive Control DNA (Heterozygous)	-20ºC
Negative Control (DNase- RNase-free H_2O)	-20ºC
DNA ladder	-20ºC

Preparation for DNA isolation and PCR

Set heat block or water bath to 95ºC

Instruct the students in cheek cell collection step to make sure proper technique and timing is used to ensure sufficient collection for DNA isolation.

Thaw 2x PCR Master Mix on ice. **Before opening tube, spin 10 sec at 6,000 rpm or greater in a microcentrifuge. Vortex 10 seconds, then spin again for 10 seconds.**

Aliquot the 2X PCR Master Mix as necessary *after* doing the above preparation.

Each package contains enough PCR 2X Master Mix for 30 reactions. Use 10 ul of 2X PCR Master Mix with 10 ul template for a final PCR volume of 20 ul.

Up to 3 positive control materials are available for PCR reactions (10 ul from Positive Control tube + 10 ul 2X PCR Master Mix for one reaction).

Run 1 or up to 3 negative control reactions.

Electrophoresis

Electrophoresis reagents are not provided in the kit. Please refer to the required materials list.

Best results are obtained by adding DNA dye (i.e. Gel Red or Sybr Safe) to molten agarose.

Avoid exposing the agarose gel to light. It is best to store and run the gel in a dark room, or cover the gel with a box during gel polymerization and the whole electrophoresis process.

There is enough DNA ladder to load 3 lanes with 10 ul.

After PCR, load 10 ul of positive control reaction for up to 6 lanes.

After PCR, load 10 ul of negative control reaction for up to 6 lanes.

After PCR, load as much as 20 ul of student PCR reaction into a lane.

Shipping, Storage and Safety

Shipping and Storage

EduPrimer™ VNTR DNA Profiling kits are shipped on blue ice. Components should be stored at temperatures shown in the above table. At proper storage conditions, components are stable for 1 year from the date received. Expiration dates are also noted on product labels.

Safety Warnings and Precautions

This product is intended for research use only. It is not recommended or intended for the diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. Consider all chemicals as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products. Wear suitable protective clothing such as laboratory overalls, safety glasses, and gloves. Exercise caution to avoid contact with skin or eyes: if contact should occur, wash immediately with water (Material Safety Data Sheet for products is available upon request).

Suggestions for Teachers

This kit is technical in nature and an excellent tool for teaching techniques, and has real links to DNA Forensics. Students will enjoy extracting their own DNA and seeing their individual band separation. Should you want to, there are other ways to spice things up even more. Here are a few possible suggestions:

Where's Waldo?

1. Determine your own band pattern by running a gel prior to class or from a previous year
2. Either post a picture or use the ladder to draw the bands locations on the board
3. Have the students know where they are in the gel by numbers
4. Make a cheat sheet with where the students are and where "Waldo" is
4. Day of experiment run your sample on the gel somewhere in the middle
5. Let the students work in groups to analyze their band and find Waldo

Smooth Criminal

1. Secretly pick two students to be the criminal. This can be done by collecting all of the student supernatant DNA samples after students are done with them and selecting two of the tubes from the whole class. Make note of which student's samples you selected. Alternatively, collect swabs from the "criminal" students' cheek cells ahead of the class period and have them prepared alongside the rest of the class.
2. Run the "criminal" samples through the PCR process alongside the rest of the class.
3. Run the criminal samples on the same gel (or gels) as the rest of the class for easy comparison. Each PCR reaction can be split into two gels, with a 20 µl PCR product split in half into two gels.
(This will only work if the student can keep a secret and is not the obvious choice)
4. Have the students compare bands and explain the effect of "Gel smiling"
5. Optionally offer a simple reward to whomever figures out who the criminals are, as well as to participating "criminal" students.

EduPrimer™ VNTR DNA Profiling Kit Overview

The EduPrimer™ VNTR DNA Profiling Kit introduces Polymerase Chain Reaction (PCR) techniques to students, or anyone wishing to learn PCR and its uses. It contains all reagents necessary for DNA isolation and PCR. PCR is an extremely important and valuable skill to have in contemporary biological and related sciences. In this particular kit, the experiment generates varying results from person to person, demonstrating the basis for the process of creating DNA profiles that are used to differentiate one person from another. After completing this experiment, one should be able to proficiently perform PCR and understand the concepts behind it.

Kit Components and Storage Conditions

(for a lab of 24 students)

Component	Amount (30 rxn's)	Storage
Solution A	6 mL	Room temp.
Solution B	0.6 mL	Room temp.
Cotton Swabs	28	Room temp.
2X PCR Master Mix	300 µL	-20ºC
Positive Control DNA (Heterozygous)	30 µL	-20ºC
Negative Control	30 µL	-20ºC
DNA ladder	30 µL	-20ºC

Additional Required Materials

- Thermal Cycler
- Heat Block or (heat plate, Beaker with de-ionized water; water bath, Tube floater; Thermometer)
- Microcentrifuge
- Microcentrifuge tubes
- Vortex
- Micropipettes (p10, p200, p1000)
- Pipette tips
- PCR tubes
- Tube Racks
- Ethanol or ethanol wipes
- Electrophoresis equipment
- Electrophoresis supplies: agarose, TBE, DNA loading buffer, running buffer, gel dye (eg. Sybr safe, Gel Red)
- Scissors and tweezers
- UV light box or "Gel Doc" equipment and program

Introduction: Theory and Background

Every one of us is different. This is true on many different levels, but it's an interesting point down on the genetic level. In fact, a large portion of our genetic makeup from one person to the next might differ by as little as 0.1%! Our genome consists of over 3 billion base pairs, though, and even that small percentage makes a big difference.

This is important in DNA profiling. There are a number of specific regions on our genomes that we can rely on to vary between individuals. For this experiment, we will be focusing on a "Variable Number Tandem Repeat" region. Throughout our DNA we've found segments that feature small repeating sequences of DNA. The repeating sequence is generally the same between individuals, but the number of times it repeats can vary. Analyze enough of these segments, and you can generate a genetic "fingerprint" for an individual.

In the EduPrimer VNTR DNA Profiling kit, we focus on the Penta E "microsatellite" region, a VNTR region with a repeating sequence 2-5 base pairs long, which features a sequence of "AAAGA" that can repeat anywhere from 5 to 26 times. This means that when a region flanking and including this repeating sequence is amplified by PCR using our primers the region will be between 246 – 351 base pairs long. Each individual will have two bands on the final gel, one inherited from each of their parents, apart from individuals who might have identical repeating numbered sequences from both parents.

What this means on the whole is that for a single VNTR site there can be wide degrees of variation, and it's unlikely that any two non-related persons will share the same band pattern. In a real world situation for DNA profiling or fingerprinting many variable regions would be used to be able to accurately distinguish one individual.

EduPrimer™ VNTR DNA Profiling Kit: Protocol

Preparation

1. Set heat block or water bath to 95ºC. For a heat block, it is recommended to add water or sand to ensure proper heat transfer. For a water bath, be sure tubes are tightly sealed and not fully submerged to avoid contamination.
2. Thaw 2x PCR Master Mix on ice. **Before opening tube, spin 10 sec at 6,000 rpm or greater in a microcentrifuge. Vortex 10 seconds, then spin again for 10 seconds.**

DNA Preparation

Do not eat or brush teeth one hour prior to cheek cell collection. Wear gloves and handle solutions carefully

1. Add 200µl of **Solution A** (red label) to a marked microcentrifuge tube.
2. **Thoroughly** roll provided cotton swab inside cheek for no less than **10 seconds** to collect cheek cells.
3. Place swab into marked tube with Solution A.
4. Cut swab above tube to a length that will fit inside the tube. Make certain the cap will shut tightly.
5. Press the tube against the vortex machine to thoroughly mix the sample, for at least 10 seconds. *Solution A contains components which chemically disrupt cell membranes and begin to unravel proteins. Under these conditions, the cheek cells will begin to lyse or break open, spilling cell contents into the solution in the tube.*
6. Place sample in heat block to incubate at 95°C for 5 minutes. Immediately place tube in ice until ready for next step.*This process continues to destroy proteins, particularly, those that damage DNA.*
7. Load sample into microcentrifuge (mini centrifuge works as well), taking care that there is another sample directly across from your sample to keep the centrifuge in balance as it spins. Close internal and external centrifuge lids.
8. Spin briefly (~10 seconds) to pool condensation that has collected on the cap.
9. Remove swab with tweezers (tweezers should be rinsed with ethanol between samples to prevent contamination).
10. Add 20 µl **Solution B** (green label) to the sample tube. *Solution B neutralizes the harsh conditions needed for lysis, preparing the solution for DNA isolation and PCR to follow.*
11. Close the tube lid tightly and vortex to mix for at least 10 seconds.
12. Load sample into microcentrifuge with the tube hinge pointing out, balancing out your sample tube, and closing the lids.
13. Spin sample for 1 minute at 12,000 rpm.
14. Look for a small clear round pellet near the bottom of the tube under the hinge. This pellet contains cellular debris. The aqueous solution (supernatant) that has not precipitated into the pellet contains cellular DNA.

PCR Reaction

*****Wear gloves and handle solutions carefully*

1. Prepare and label a small PCR tube with your name. Label both the top and side of the PCR tube to ensure clarity.
2. Ensure that the "2x PCR Master Mix" is on ice and has been spun at 6,000 RPM or greater in a microcentrifuge for 10 seconds, vortexed for 10 seconds, then spun again for 10 seconds before opening the Master Mix tube.
3. (as directed by instructor) Label and prepare PCR tubes for controls. Follow the table below
4. Add 10 µl of "2X PCR Master Mix" and 10 µl of supernatant from step 14 above (avoid the pellet) to the labeled PCR tube for a total of 20 µl as indicated in the table below. *The supernatant contains your genomic DNA which will become the template for polymerase chain reaction (PCR.)*(Note: It is preferred that the PCR reaction mix preparation is done on ice).
5. Mix the 20 µl PCR reaction mixture by pipetting in and out with the pipette, and the close the lid tightly.
6. Store the sample on ice until it is ready to be loaded into the thermal cycler.

PCR reaction mixture tables

Student Sample PCR Mixture	
2x PCR Master Mix	10 µl
Genomic Template (Supernatant)	10 µl
Volume total	20 µl

Control Samples PCR Mixture			
Positive Control		**Negative control**	
2x PCR Master Mix	10 µl	2x PCR Master Mix	10 µl
Positive control DNA	10 µl	Negative Control	10 µl
Volume total	20 µl	Volume total	20 µl

Positive and negative controls give guidelines or boundaries to the experimental results. The positive control will show a result for an individual with widely spaced bands near the upper and lower limits of this VNTR region. Negative control show the result if no DNA is present for the PCR.

DNA ladder is a standard which is used like a ruler to show how DNA of a certain length will migrate through a gel during electrophoresis.

PCR Parameters

Program your thermal cycler as follows:

1. 94ºC – 2 minutes
2. 94ºC denaturing – 20 seconds}
3. 65ºC annealing – 20 seconds} **repeat steps 2, 3, & 4 for 40 cycles**
4. 72ºC extension – 30 seconds}
5. 72ºC – 5 minutes
6. 4ºC – finished / hold

Agarose Gel Electrophoresis

General Procedure, detailed directions as given by instructor

- Prepare 0.8 – 1% agarose.
- Set up electrophoresis apparatus and pour the 1% molten agarose for gelation.
- For staining, use a DNA dye which is added directly to the molten agarose. For light sensitive dyes, keep the gel in the dark during gelation, either by performing in a dark room or placing a box over the gel.
- Use at least 10 µL of PCR product to visualize results by electrophoresis on agarose gel. If gel well volume will accommodate more than 10 ul, a higher volume is preferred. Loading dye is added to the sample to ensure that the sample will sink to the bottom of the well and properly enter the agarose gel.
- Mix sample with loading dye according to instructor directions before carefully pipetting into a well in the gel. Be sure to record which well holds your sample.
- Run at ~100V for ~20 minutes and stop before loading dye has run off gel. Depending on the DNA dye used, caution may need to be taken to reduce exposure of gel to light.
- Visualize under UV and record the results manually or by photography
- Compare individual experimental bands to positive control DNA.

Example of Gel Setup and Loading: Run 3 gels with 12 wells each to accommodate 24 students and 6 control reactions.

Lane 1:	10 µl DNA Ladder
Lane 2:	10 µl Negative Control
Lane 3:	10 µl Positive Control
Lanes 4 -11	Up to 20 µl of each student sample

Results and Discussion

Compare your results to your fellow classmates. Is your band pattern different from others? What causes this?

In what ways is this information useful?

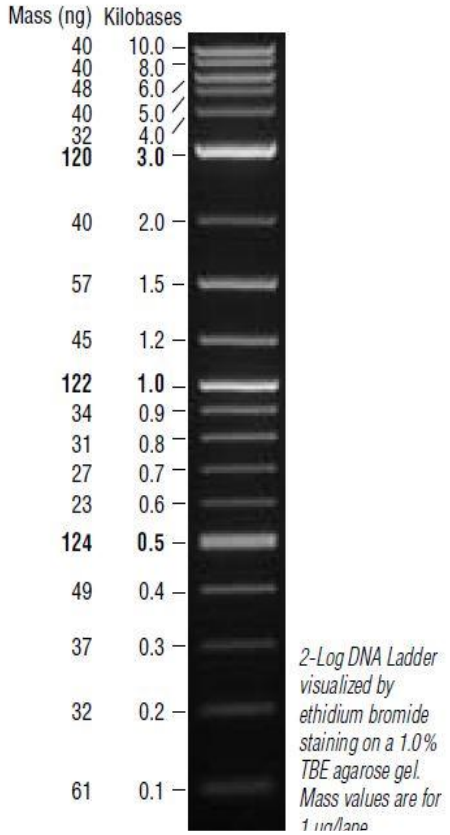

Fig 1. 10K DNA Ladder

DNA Ladder reference for band size comparison

Data Analysis and Class Exercise

Class Allele Frequency

The results from the whole class can be analyzed to determine the allele frequency within the class. Record the results in the table below.

Observed Class Genotypes

Genotypes	# of individuals	# of alleles	frequency
(+)/(+) homozygous w/ Alu			# of (+) alleles . Total (+) & (-) alleles
(+)/(-) heterozygous		(+) (-)	
(-)/(-) homozygous w/out Alu			# of (-) alleles . Total (+) & (-) alleles
	Sum= class total	Sum= # of alleles	Sum = 1.00

Each person has two alleles, consequently, in calculating allele frequency, both copies are taken into account.

To determine the frequency of the allele with the *Alu* insert, double the number of individuals with (+)/(+) homozygous genotypes and add the number of individuals with (+)/(-) heterozygous genotypes. This sum accounts for all the (+) alleles.

To determine the frequency of the allele without the *Alu* insert, double the number of individuals with (-)/(-) homozygous genotypes and add the number of individuals with (+)/(-) heterozygous genotypes. This sum accounts for all the (-) alleles. Divide this sum

Compare with classes around the world:

Scientists use *Alu* frequency to study human populations. To learn examples and compare the frequencies measured in your class to other classes and populations around the world, visit the "Allele Server" in Cold Spring Harbor's Dolan DNA Learning Center at www.bioservers.org/bioserver/.

EduPrimer™ DNA Profiling Kit: Background

Introduction to PCR

In 1983, during his time working at Cetus Corporation, Kary Mullis developed a technique that has changed the field of genetics and biological science in general. This revolutionary process was termed “polymerase chain reaction,” or PCR. He earned the Nobel Prize in Chemistry in 1993 due to his innovation. His technique enabled researchers, besides a few expert microbiologists, to amplify DNA. Before that, amplification of DNA was extremely difficult and time consuming. Now, scientists in any field can incorporate molecular biology into their research with PCR.

Currently, PCR is used in a wide variety of areas, for example, gene mapping, DNA sequencing, gene expression, gene detection, forensics, criminal investigation, medical diagnostics, and genome sequencing. Very few of these applications were practically possible before PCR. The experiment does require an initial investment in specially made machinery, but with the proper equipment, nearly anyone can perform a successful PCR experiment without significant cost.

PCR and Biotechnology — Revolutionizes an Entire Research Community

PCR is capable of producing large amounts of targeted DNA from an extremely small amount of starting material, known as your template. DNA can be obtained from any cell such as blood cells, hair cells, cheek cells etc., and after proper treatment to isolate DNA, PCR can be applied to create millions of copies of virtually any desired DNA sequence. That is one of the most significant powers of PCR, specificity. Although you may put an entire genome worth of DNA into a PCR, it amplifies the exact piece of DNA desired and leaves the rest out.

The basic components of PCR:

- Reaction Buffer
- DNA nucleotides (dNTP’s) of each adenine, guanine, thymine and cytosine
- DNA polymerase
- 2 DNA oligonucleotide primers
- Template DNA (starting material)

PCR Makes Use of Two Basic Processes in Molecular Genetics

1. **Complementary DNA strand hybridization**

For DNA to be amplified, one must have a known sequence which flanks the gene of interest upstream and downstream. These sequences are used to create what are known as

'oligonucleotide primers,' meaning a short ~20 base pair nucleotide sequence which is used as a starting point for DNA replication. The primers are said to be complementary to their target regions, so they will anneal (attach) to those regions specifically. Primers are required by DNA polymerase because it cannot add nucleotides without a preexisting chain.

Complementary Strand Hybridization occurs when two different oligonucleotide primers anneal to each of their respective complementary base pair sequences on the template. They are designed specifically to anneal at opposite ends of opposite strands of the specific sequence of DNA that is desired to be amplified.

2. DNA strand synthesis via DNA polymerase

In a PCR, a special type of DNA polymerase is used that is able to withstand the temperature fluctuations required for thermal cycling. Most DNA polymerases cannot tolerate the high temperatures and fluctuations from ~60ºC-94ºC. The breakthrough in PCR came with the isolation of DNA polymerase from a thermophilic bacterium known as Thermus aquaticus. This bacterial species lives in high temperature steam vents and therefore its DNA polymerase evolved to withstand extremely high temperatures.

During PCR, DNA is synthesized and multiplies by 2 each cycle, thus the growth of DNA copy # over the reaction is exponential. In theory, after 30 cycles there will be 2^{30}. This is over a billion copies of DNA. Yielding this amount of DNA allows the possibility of visualization through a variety of means. One of the most popular visualization methods is agarose gel electrophoresis.

Genes and DNA

The human genome contains 23 pairs of chromosomes that contain a total of thirty to fifty thousand genes, most of which generally code for proteins. However, those genes only comprise about 5% of the genome, leaving 95% as so-called non-coding DNA. This noncoding DNA is found not only between, but within genes, splitting them into segments. In eukaryotes, non-coding DNA found within genes is known as **introns**. The sequences that do code for proteins are called **exons**. In eukaryotes, genomic DNA is transcribed into RNA molecules containing both introns and exons for a particular gene. While the RNA is still in the nucleus (before being transported out of the nucleus), the introns (in = stay within the nucleus) must be removed from the RNA while the exons (ex = exit the nucleus) are spliced together to form the complete coding sequence which will soon be translated into the protein. This process is called RNA splicing. Some genes may contain a few introns, others may contain dozens. Interestingly, it is the non-coding 'junk' DNA that is useful to us when considering a DNA profile of an individual, instead of the DNA that actually codes for life.

As discussed, functional segments of genes (exons) code for proteins. Proteins are molecules that carry out most cellular functions. Exon sequences are therefore very similar among individuals, because even slight difference can change the function of the protein in a

potentially harmful way (many diseases are caused by mutated proteins). Introns, however, often vary in size and number among individuals. Intron sequences are thought to be the result of the differential accumulation of mutations throughout evolution that are silently passed to descendants through the hereditary code. It is this difference in intron sequences that allows us to determine human genetic diversity. The identification of these distinctive characteristics in DNA represents the molecular basis for human identification and population genetics. Throughout evolution, intron sequences have been the target of random insertions by short repetitive interspersed elements, also known as SINEs. SINEs have become randomly inserted within our introns over millions of years.

One such repetitive element is called the **Alu element** (Figure 1). The Alu element is a DNA sequence about 300 base pairs long that is repeated; one copy at a time, almost 500,000 times within the human genome. The origin and function of such randomly repeated sequences is not yet known. The Alu name comes from the Alu I restriction enzyme (enzymes that cut DNA at specific sequences) recognition site that is found in this sequence. The following section reviews the Alu element in more detail.

PCR Stages

The machinery required to perform PCR is known as a thermal cycler. The thermal cycler enables the steps of PCR to be automated. The reaction involves a repetitive series of cycles, each of which consists of template denaturation, primer annealing, and extension of the annealed primer by Taq DNA polymerase. Before beginning DNA amplification, genomic DNA is prepared from students' cells. The students' DNA is then added to a mixture of the necessary reagents: oligonucleotide primers, thermostable DNA polymerase (Taq), the four deoxynucleotides (A, T, G, C), and reaction buffer. These reagents are pre-mixed as a 2X PCR Master Mix in the EduPrimer™ DNA profiling kit. The tubes are placed into the thermal cycler. These thermal cyclers contain an aluminum block that holds the samples and can be rapidly heated and cooled across extreme temperature differences. The rapid heating and cooling of this thermal block is called temperature cycling or thermal cycling.

The first step of the PCR temperature cycling procedure involves heating the sample to 94°C. At this high temperature, the template strands separate. This is called the denaturation step.

The thermal cycler then rapidly cools to 60°C to allow the primers to anneal to the separated template strands. This is called the annealing step. The two original template strands may reanneal to each other or compete with the primers for the primers complementary binding sites. However, the primers are added in excess such that the primers actually out-compete the original DNA strands for the primers' complementary binding sites.

Lastly, the thermal cycler heats the sample to 72°C for Taq DNA polymerase to extend the primers and make complete copies of each template DNA strand. This is called the extension step. Taq polymerase works most efficiently at this temperature. Two new copies of

each complementary strand are created. There are now two sets of double-stranded DNA (dsDNA). These two sets of dsDNA can now be used for another cycle and subsequent strand synthesis. At this stage, a complete temperature cycle (thermal cycle) has been completed.

Each step takes about 30 seconds to 1 minute, and this process continues for roughly 30-40 cycles depending on how the user has programmed the thermal cycler. Each step is repeated in that order each cycle until it is completed. At the end, the product is put on hold at a low temperature, generally 4°C, until the user is ready to proceed to the analysis of the product.

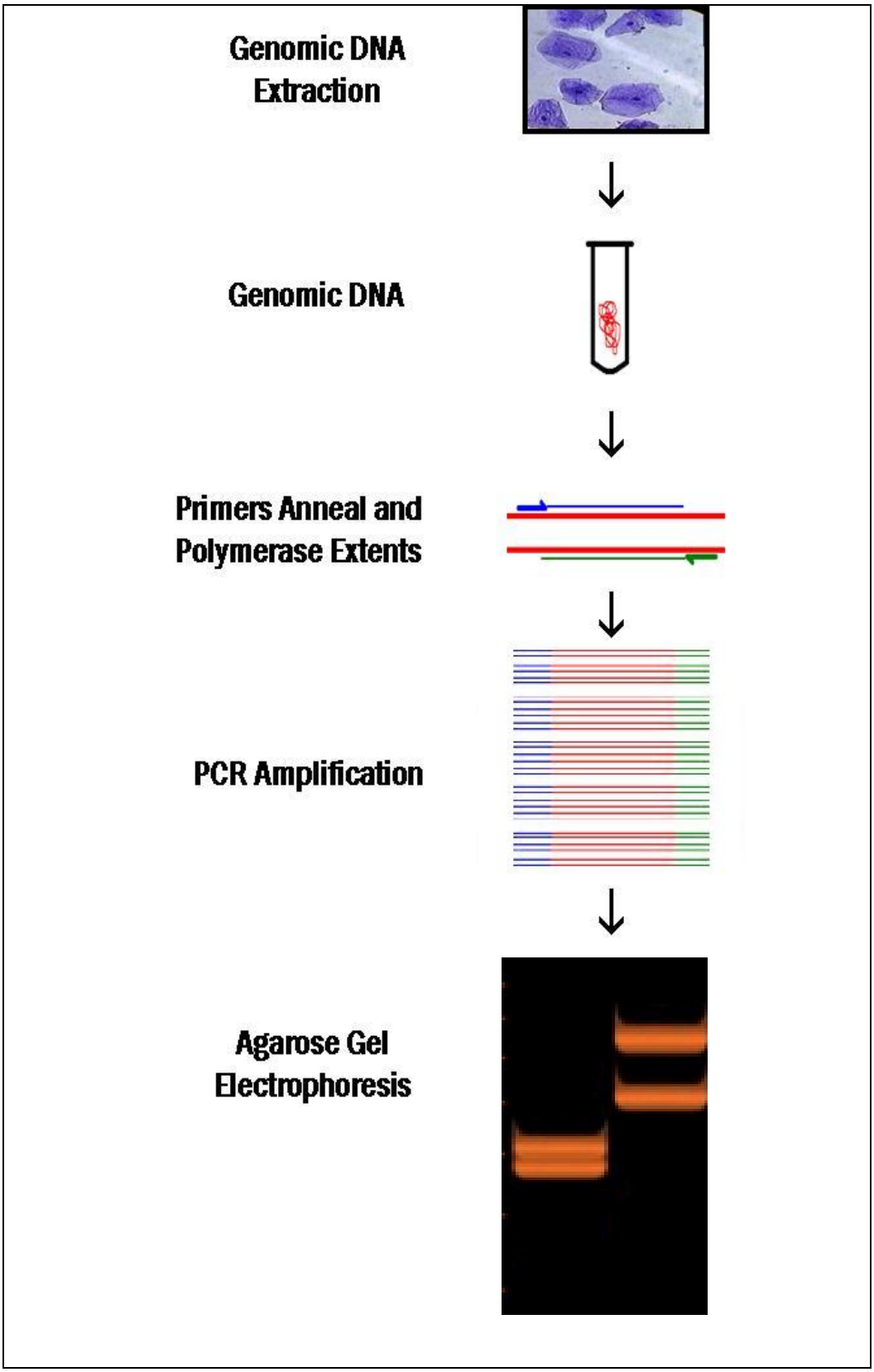

Figure 3. Experiment flowchart from start to finish

Troubleshooting

Symptom	Possible causes	Solutions
No amplification product	Questionable template quality	Analyze starting material
	Inhibitory Substance in reaction	Decrease sample volume
	Insufficient cycle #	Run additional cycle
	Incorrect thermal cycler program	Verify times and temperatures
	Errors in heat block incubation	Calibrate heating block, use sand or water to maximize contact with tube for proper heat transfer
	Contaminated tubes/solutions	Autoclave tubes and use filter tips
	Primer annealing temperature too high	Lower annealing temperature in 2º increments
Weak bands/faint signal	Low concentration of DNA template	Increase swabbing time, thoroughly swab.
	DNA Dye degradation during preparation	Light sensitive dyes should be kept in the dark during gel preparation. Prepare in dark room or place a box over the electrophoresis apparatus during gelation and electrophoresis.
	Expired, contaminated or degraded DNA dye	Verify that the DNA dye has not degraded in storage, been contaminated or expired.
Non-specific amplification product	Premature Taq-polymerase replication	Mix solutions on ice, place rxn directly to 94º thermal cycler
	Primer annealing temperature too low	Raise annealing temperature in 2º increments
	Insufficient mixing of reaction solution	Mix solutions thoroughly before beginning the reaction
	Exogenous DNA contamination	-Wear gloves -Use dedicated area for sample preparation -Use non-aerosol tips

Technical Service

For more information or technical assistance, please call, write, fax, or email.

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